FOLIA MEDICA CRACOVIENSIA
Vol. LV, 1, 2015: 43–51
PL ISSN 0015-5616

Inhibition of myeloperoxidase activity have impact on the formation of DNA double-strand breaks induced by etoposide in HL-60 cell line

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Abstract: Many studies have shown the role of myeloperoxidase (MPO) in leukemogenic activity of etoposide. The aim of our study was to determine whether inhibition of MPO activity has influence on the formation of double-stranded DNA breaks (DSBs) that may contribute to the characteristic of leukemia translocations. Studies were carried out on HL-60 cell line, which were preincubated with the MPO inhibitor 4-aminobenzoic acid hydrazide (ABAH), or antioxidant N-acetyl-L-cysteine (NAC), followed by incubation at different concentrations of etoposide (1–10 μM) for 4 hours. Cytotoxicity was investigated using propidium iodide staining. Marker of DSBs, a phosphorylated form of histone 2AX (γH2AX) was detected using immunocytochemical methods. Cells were analyzed by flow cytometry. ABAH significantly reduced the cytotoxicity and the γH2AX level induced by lower concentrations of etoposide (1 and 1.5 μM) and did not modify the action of higher concentration (10 μM) of this cytostatic drug. NAC exerted similar impact as ABAH on the level of γH2AX induced by etoposide. The results of this study suggest that MPO contributes to increase of the DSBs level induced by low concentrations of etoposide in myeloid cells.

Key words: etoposide, myeloperoxidase, 4-aminobenzoic acid hydrazide, double-stranded DNA breaks, γH2AX.

Introduction

Double-stranded DNA breaks (DSBs) are considered the most lethal damage of nucleic acid. Unrepaired breaks lead to apoptosis and chromosomal aberration and contribute to neoplastic transformation. Their role in carcinogenesis indicates drug-dependent tumors occurring after the treatment with DNA damaging agents, which induce DSBs [1, 2]. This

damage can also arise under the influence of reactive oxygen species (ROS), whose level is often elevated in certain cancers, including myeloid malignancies [3]. Mutations in proto-oncogenes and fused genes characteristic of leukemias contribute to the overproduction of ROS. In turn, ROS can directly lead to the formation of DSBs [3, 4]. It was observed that acute myeloid leukemia (AML) cells with a mutation of the FMS-like tyrosine kinase-3/internal tandem duplication (FLT3/ITD), characteristic of aggressive forms of the disease, produce more ROS and DSBs [4]. In addition, blocking FLT3 using inhibitor CEP-701 led to decreased level of DSBs, which was detected with the marker of this damage, phosphorylated histone 2AX (γH2AX).

In a very short time after the formation of DSBs, H2AX is phosphorylated by the ataxia teleangiectasia mutated (ATM) kinase and accumulates at the site of DNA damage. Phosphorylated H2AX takes part in response to DNA damage, inter alia by activating DNA repair proteins [5, 6].

DSBs are repaired in two ways: homologous recombination (HR) and nonhomologous end joining (NHEJ) [3, 7]. NHEJ repair takes place in all cell cycle phases and is often used to repair DSBs [8]. The process of NHEJ connecting broken DNA ends with a very low homology is not always precise and often leads to small DNA deletions [9, 10], which can cause chromosomal translocations, so very obvious for leukemias. Studies have shown that these translocations occur during the repair of DSBs in the NHEJ or in variable, diverse and joining V(D)J recombination [11–13].

One of the chemotherapeutic agents which induce DSBs in cancer cells and in normal proliferating cells is etoposide [14]. Etoposide is widely used in the treatment of certain solid tumors, leukemias and lymphomas [15]. The most common side effect caused by this drug is myelosuppression [16, 17]. Etoposide can also induce treatment-related acute myeloid leukemia (t-AML) in a few percent of patients [18]. DSBs that arise after etoposide treatment may be not only due to the stabilization of the topoisomerase II–DNA cleavable complex but also due to its radicals produced in myeloid precursors under the influence of myeloperoxidase (MPO) [14, 19]. It was also shown that the appearance of translocation of mixed-lineage leukemia (*MLL*) gene, a mutation characteristic of t-AML, correlates with the level of etoposide phenoxyl radicals in myeloid progenitor cells expressing the CD34 antigen [20].

Etoposide can also induce oxidative damage to DNA and single strand breaks (SSBs) that interfere with the activity of DNA topoisomerase II in MPO-rich cells [21, 22]. Examining the role of MPO in the formation of DSBs under the influence of etoposide can contribute to the understanding of the leukemogenic activity of etoposide. The aim of our study was to verify the hypothesis whether the inhibition of MPO activity can influence the DSBs caused by etoposide in cells of myeloid lineage.

Materials and methods

HL-60 Cell culture

HL-60 cell line was purchased from Europea Branch of ATCC (American Type Cell Culture, UK). The cells were grown in RPMI 1640 medium (ATCC, UK) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), without antibiotics at 37°C

in a 5% CO_2 and 95% humidified atmosphere. The cells in the logarithmic growth phase were seeded in 24-well culture plates at a density of 0.4×10^6 cells/well.

Treatment of HL-60 cells

In order to determine the role of MPO in cytogenotoxicity of etoposide, the cells (0.4×10^6 cells/well) were preincubated with 100 μM specific inhibitor of MPO 4-aminobenzoic acid hydrazide (ABAH) for 24 hours followed by incubation in the presence of different concentrations of etoposide (1–10 μM) for the next 4 hours. We used 100 μM ABAH because further increasing the concentration of this inhibitor did not inhibit more of MPO activity (data not shown). ABAH and etoposide were dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted in phosphate buffered saline (PBS). Final concentration of DMSO did not exceed 0.3% in the culture medium. Reagents were purchased from Sigma-Aldrich, St. Louis, Missouri, USA. Control cells were incubated in the presence of DMSO only. The experiment was repeated three times.

Evaluation of cytotoxicity and detection of γH2AX

To determine the cytotoxicity, HL-60 cells after the treatment were washed twice in PBS ($230 \times g$) for 5 minutes. The cells were resuspended in 100 μL of PBS and 10 μL of propidium iodide (PI, Sigma-Aldrich, St. Louis, MO, USA) staining solution (10 μg/mL) was added. The cells were incubated for 1 minute in the dark. Next, 300 μL of PBS was added and the cells were immediately analyzed by flow cytometry.

For the detection of DSB, phosphorylated H2AX (γH2AX) at serine 139 (Santa Cruz Biotechnology, Inc. Dallas, Texas, USA) was detected as a marker [23, 24]. The cells were fixed on ice with 1% methanol-free formaldehyde solution in PBS for 15 minutes and permeabilized with ice-cold 70% ethanol in deionized water for 24 hours. After washing, the cells (0.4×10^6) were stained with primary rabbit monoclonal anti-γH2AX (Ser 139) antibody dissolved in Bovine serum albumin — Triton X 100 — PBS medium (BSA-T-PBS, Sigma–Aldrich Co. St. Louis, MO, USA) for 2 hours at room temperature. Next, they were washed with BSA-T-PBS ($300 \times g$, 5 minutes) and incubated with the secondary anti-rabbit IgG (H+L) $F(ab')_2$ fragment of goat antibody conjugated with Alexa Fluor 647 (Santa Cruz Biotechnology, Inc. Dallas, Texas, USA), diluted in BSA-T-PBS for 30 minutes at room temperature in the dark. An isotype control was the rabbit monoclonal IgG XP antibody (Santa Cruz Biotechnology, Inc. Dallas, Texas, USA). DNA was stained with 5 μg/mL PI and 0.1% RNAse A (Sigma–Aldrich Co. St. Louis, MO, USA) in PBS for 30 minutes at room temperature in dark. Red and blue lasers were used for the analysis of the cells, using 633 nm excitation for Alexa Fluor 647 (660/20 BP filter) and 488 nm excitation for PI/PE (575/26 BP filter). Cell doublets and debris were excluded from analysis using PI width versus PI area. Analysis was performed on at least 20.000 single cells.

Cytochemical detection of MPO activity

MPO activity was detected using 4-chloro-1-naphthol kit (Merck KgaA, Darmstadt, Germany) according to manufacturer's instruction. The cytochemical reaction is based on the oxidation of 4-chloro-1-naphthol which is converted to a black brownish insoluble dye. The intensity of staining is proportional to MPO activity.

Statistical analysis

One-way ANOVA and the Newman–Keuls post-hoc test were used to calculate the statistical significance between groups. In the absence of the homogeneity of variance, the non-parametric Kruskal–Wallis test was used. All data are shown as mean ± SEM.

Results

MPO inhibitor reduced the cytogenotoxic effects induced by low concentrations of etoposide

Etoposide increased significantly the number of dead cells compared to controls, depending on the concentration used (Fig. 1). An inverse relationship was observed in the case of γH2AX fluorescence intensity. Greater increase in γH2AX fluorescence was observed under the influence of lower concentrations of etoposide (1 and 2 μM), than when 10 μM of the cytostatic drug was used (Fig. 2, 3). Given that apoptosis is preceded by DNA damage, this effect is likely due to apoptosis of the cells after 4 hours of incubation with 10 μM of etoposide compared to the low concentrations (1–2 μM), where DNA damage just started to happen.

Preincubation with ABAH followed by incubation with etoposide led to significant reduction of the proportion of death cells treated with low concentrations of etoposide (1 or 1.5 μM) compared to etoposide alone (Fig. 1). However, more death cells were significantly observed after treatment with ABAH and etoposide (1 or 1.5 μM) compared to the control. ABAH did not reduce the cytotoxic effects of etoposide at highest concentration (10 μM) (Fig. 1).

Preincubation with ABAH followed by treatment with 1 μM of etoposide significantly decreased the fluorescence intensity of γH2AX compared to etoposide alone (Fig. 2, 4). However, the level of H2AX phosphorylation was statistically significant and higher after treatment with ABAH plus 1 μM of etoposide in comparison to the control. Treatment with ABAH and 2 μM of etoposide led to an insignificant but a clear reduction in the fluorescence intensity of γH2AX compared to the cytotoxic drug alone. ABAH did not modify significantly the level of H2AX phosphorylation induced by etoposide at a concentration of 10 μM, although there was an increase in the intensity of γH2AX fluorescence after treatment with ABAH and etoposide (Fig. 2).

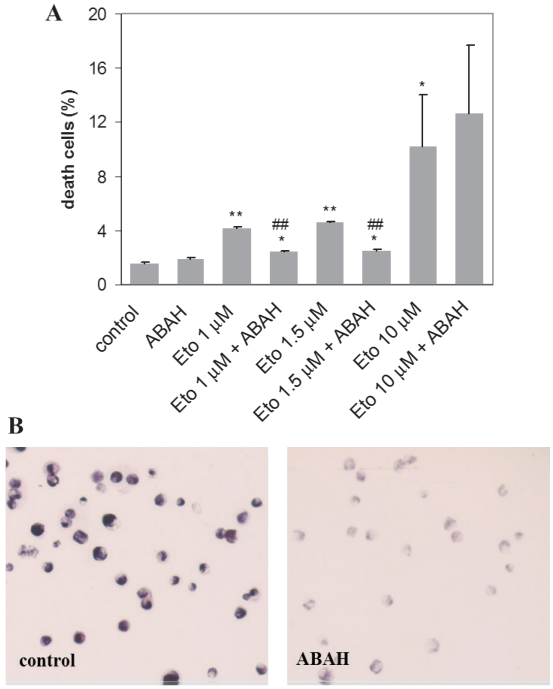

Fig. 1. The cytotoxicity induced by etoposide in HL-60 cells with normal or decreased activity of MPO. The cells were pretreated or not with 100 μM ABAH for 24 hours followed by incubation with etoposide. Cytotoxicity was analyzed by flow cytometry. (A) Precentage of death cells. Data are presented as mean ± S.D. of the mean. Eto — etoposide, *p <0.05, **p <0.01 vs. control, ##p <0.01 vs. Eto. (B) Cytochemical detection of MPO activity in control and ABAH-treated HL-60 cells. × 400.

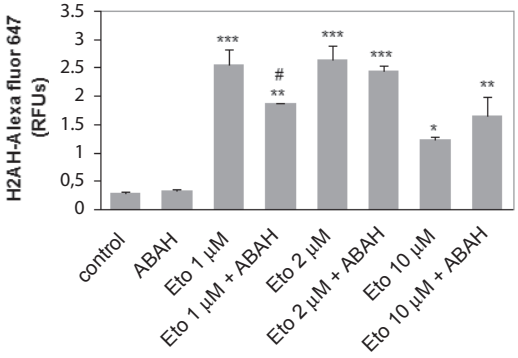

Fig. 2. The influence of etoposide on phosphorylation of H2AX in HL-60 cells with normal or decreased activity of MPO. The cells were pretreated or not with 100 μM ABAH for 24 hours followed by incubation with etoposide. Fluorescence of γH2AX was analyzed by flow cytometry. Data are presented as mean ± S.D. of the mean. Eto — etoposide, *p <0.05, **p <0.01, ***p <0.001 vs. control, #p <0.05 vs. Eto.

Antioxidant NAC protects HL-60 cells against etoposide-induced phosphorylation of H2AX

To confirm that etoposide prooxidative action leads to increased phosphorylation of H2AX, we performed preincubation of HL-60 cells with NAC followed by incubation with etoposide. As in the case of treatment with ABAH, preincubation with NAC significantly decreased the level of γH2AX induced by 1 μM of etoposide compared to the cytostatic drug alone (Fig. 3, 4). After treatment with NAC followed by 2 μM of etoposide, the level of γH2AX decreased but not to a significant level compared to the cytostatic drug alone. However, NAC did not significantly modify the impact of 10 μM etoposide on the level of H2AX phosphorylation (Fig. 3).

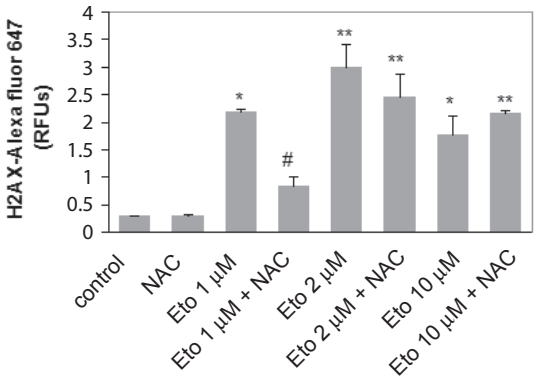

Fig. 3. The influence of free radicals on etoposide induced phosphorylation of H2AX in HL-60 cells. The cells were pretreated or not with 6 mM NAC for 1 hour followed by incubation with etoposide. Fluorescence of γH2AX was analyzed by flow cytometry. Data are presented as mean ± S.D. of the mean. Eto — etoposide, *p <0.05, **p < 0.01, ***p <0.001 vs. control, #p <0.05 vs. Eto.

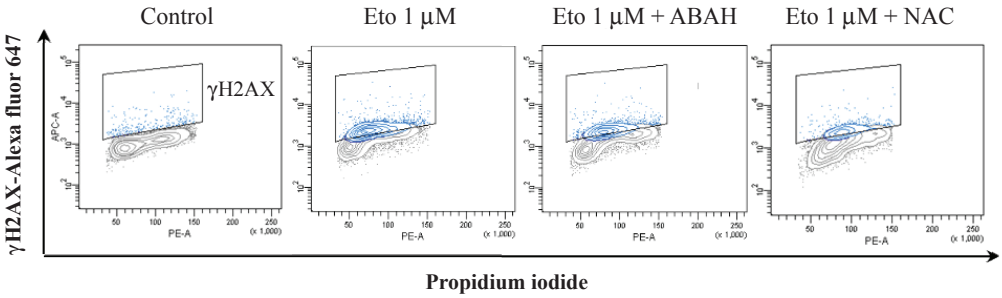

Fig. 4. Representative contour plots of γH2AX and DNA staining of HL-60 cells. Eto — etoposide. γH2AX is located within rhomboid gates.

Discussion

There is increasing evidence for the role of MPO in inducing the carcinogenic effect of etoposide [20, 25]. Etoposide induces myeloid leukemia or myelodysplastic syndromes and does not lead to the development of other types of white blood malignancies [16]. Etoposide and its metabolites catechols are subjected to a one-electron oxidation by MPO to much less genotoxic radicals [19], as has been identified by electron paramagnetic resonance [25].

Our results indicate that inhibition of MPO activity decreases the lethal and mutagenic DSBs induced by the low concentrations of etoposide in HL-60 cells. MPO may, therefore, increase the cytogenotoxic effect of lower concentrations of this cytostatic drug. Our study is probably the first to point out the correlation between the role of inhibition of MPO activity in reducing the level of the DSB and the concentration of etoposide. It should be added that the risk of t-AML increases with increasing frequency of etoposide use or length of use [26].

The formation of DSBs after the treatment of HL-60 cells with a low concentration of etoposide (1 μM) is much more dependent on the prooxidative action of this cytostatic drug compared to a higher dose. ABAH limited H2AX phosphorylation induced by 1 μM of etoposide by about 30%, while NAC by about 60%. It is worth noting that NAC decreased etoposide-indued phosphorylation of H2AX to an insignificant level compared to the control.

However, after incubation with ABAH and 1 μM of etoposide, a significantly higher level of γH2AX was observed compared to the control. This effect could be due to the incomplete inhibition of MPO through ABAH [27]. Moreover, although MPO is a major enzyme that metabolizes etoposide in myeloid cells, because the level of expression of cytochrome P450 is much lower in these cells, the fact that prostaglandin synthetase is also involved in the formation of etoposide radicals [19] cannot be ruled out.

Our research shows that the higher the concentration of etoposide, the lesser the role of MPO in the induction of DSBs. Formation of DSBs induced by 10 μM of etoposide in myeloid cells is caused most likely through direct interaction with a complex of DNA–topoisomerase II. Indirectly, it can be concluded that the participation of MPO in genotoxic action at a low concentration of etoposide correlated with the induction of cytotoxicity. ABAH restricted the cytotoxic effects of etoposide at low concentrations (1 and 1.5 μM) but not at high concentration. It should be added that etoposide was detected at a concentration of 0.2–150 μM [16, 28–32] in the serum of patients with AML.

Etoposide can be a source of radicals damaging macromolecules not only DNA but also proteins, including DNA repair proteins, leading to various kinds of damage such as DSBs, increasing the likelihood of translocation and mutagenesis in myeloid cells with highly active MPO. The obtained results indicate the role of inhibition of MPO activity in reducing the most lethal and mutagenic DNA lesions caused by low concentration of etoposide in myeloid cells.

Acknowledgements

The study was supported by the Jagiellonian University Programs No. K/ZDS/005620.

Conflict of interest

None declared.

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